ROLE OF THE NUCLEAR CORTISOL BINDING PROTEIN IN THE CONTROL OF TRANSCRIPTION OF THYMOCYTE NUCLEI BY CORTISOL

N. VAN DER MEULEN, A.D. ABRAHAM and C.E. SEKERIS
Institut für Physiologische Chemie der Philipps Universität, 355 Marburg/Lahn, West-Germany

Received 8 June 1972

1. Introduction

Evidence is accumulating that steroid hormones regulate gene activity in target cells [1, 9–11, 13, 14, 20, 22, 23, 25, 26, 30, 31].

Indirect findings suggest that the specific association of hormonal steroids with receptor proteins is instrumental in this process. The experiments presented in this paper were devised to directly assess the role of nuclear receptors in mediating hormone action.

Our earlier findings have demonstrated that glucocorticoids and androgens added directly to isolated thymus nuclei lead to inhibition of RNA synthesis [1]. We could further show that cortisol binds to a nuclear protein extracted from isolated nuclei by buffered salt solution, having a molecular weight of approx. 150 000 [2]. This receptor protein can also bind cortisol directly as shown in equilibrium dialysis experiments.

In preliminary experiments we have observed that cortisol does not inhibit RNA synthesis *in vitro*, if the receptor protein is previously extracted from the isolated thymocyte nuclei. Addition of the receptor protein to the system restored its capacity to react to cortisol. These findings are reported in detail below.

2. Materials and methods

Male Wistar BR II rats, weighing 90–100 g, were used. The animals were kept under standard conditions. Bilateral adrenalectomy of rats was performed 3 days before sacrifice. Reagent grade chemicals were used throughout. Sephadex G-25 and G-200 were purchased from Pharmacia (Uppsala) DEAE-cellulose ion exchanger from Whatman (London). Radioactive labelled [1β,2β-^{3}H] cortisol (42 or 56 Ci/mmole), and [5-^{3}H] uridine-5′-triphosphate (1 Ci/mmole) were purchased from the Radiochemical Centre, Amersham (England). Unlabelled cortisol was a kind gift of Schering A.G. (Berlin) and was dissolved in 20% ethanol.

2.1. *Preparation of nuclei and nuclear extracts*

The rats were killed by cervical dislocation, thymi were quickly removed and placed into ice-cold Tris–sucrose–salt buffer (TSS) consisting of 0.25 M sucrose 0.025 $CaCl_2$, 0.01 M $MgCl_2$ in Tris-HCl (pH 7.55) finely minced with scissors, filtered successively through 4 and 8 layers of cheese cloth and centrifuged at 750 *g* for 5 min. The thymocytes were recovered after 2 washes by centrifugation, then homogenized in 5 vol of TSS by means of a Potter–Elvehjem homogenizator with a motor driven teflon pestle and further treated with 1% Triton X-100 as described earlier [1].

Triton-nuclei were extracted with a buffer (TEM) consisting of 0.01 M Tris-HCl (pH 8.0), 0.001 M EDTA, 0.001 M β-mercaptoethanol, 0.0005 M $MgCl_2$ and 0.3 M NaCl. The nuclear extract was centrifuged at 10 000 *g* for 10 min and the supernatant used for the further experiments.

2.2. *Incubation with radioactive cortisol*

The 0.3 M NaCl-TEM extract of isolated thymus nuclei was incubated in the presence of 10^{-9} M [^{3}H] cortisol for 12 hr at 3° under continuous shaking.

North-Holland Publishing Company – Amsterdam

2.3. *Sephadex and DEAE-cellulose chromatography*

Sephadex G-200 columns were used at 3° equilibrated with the same buffer which served for the preparation and incubation of the nuclear extract. Fractions were collected and optical density monitored at 280 nm.

2.3.1. DEAE-cellulose chromatography

The labelled nuclear extract was passed through a column of Sephadex G-25 (4 × 40 cm) equilibrated in standard buffer consisting of 10 mM Tris-HCl (pH 8.3), 2 mM β-mercaptoethanol and 5% glycerin. The protein-peak was applied to DEAE-cellulose column precycled and equilibrated with the same buffer. After washing with two column-volumes the column was eluted with a gradient of 0.1–0.5 M NaCl in the same buffer.

2.4. *Linear sucrose gradient sedimentation*

A 5–20% sucrose gradient in 0.3 M NaCl-TEM buffer was formed by a gradient mixer. The sedimentation was performed in 5.5 or 11 ml cellulose nitrate tubes using a Beckman L2 ultracentrifuge and a SW 65 K or SW 41 Spinco head at 37 000 or 35 000 rpm for 17 hr, respectively. Three drop fractions were collected from the bottom of the tubes and used either for the determination of radioactivity as described previously [5] or for the functional test as follows: The fractions were preincubated with nuclear sediment (prepared as described below) in the presence or absence of 10^{-5} M cortisol for 2 hr at 3° under continuous shaking and then tested for RNA synthesizing capacity.

2.5. *Preparation of nuclear sediment*

The triton purified nuclei from adrenalectomized rats were homogenized in 10 vol of 0.05 M Tris-HCl (pH 7.9) containing 0.1 M NaCl and left at 0° for 10 min. The sediment of lysed nuclei obtained after a centrifugation at 8000 *g* for 10 min was suspended and homogenized in 0.5 ml Tris-HCl (pH 7.9) buffer. Quantitation of this preparation was performed on the basis of the DNA and protein content, determined according to Burton et al. [8] and Lowry et al. [17].

2.6. *Incubation of nuclear sediment with cortisol*

Nuclear sediment preparations (in amounts of 100–150 μg DNA) were incubated with or without unlabelled cortisol (10^{-5} M) in the presence of the cortisol binding protein fraction obtained by Sephadex G-200 or DEAE-cellulose chromatography. The mixtures were adjusted to 0.1 M NaCl and left at 0° for 1 hr before assaying for RNA-polymerase activity.

2.7. *Determination of RNA polymerase activity*

The incorporation of [^{3}H] UTP into acid precipitab material was taken as a measure of the activity of the preparation. The *in vitro* system consisted of 0.5 μmole each of ATP, GTP, CTP, 10 μg creatine phosphokinase, 6 μmoles β-mercaptoethanol and 5 μmoles Mn^{2+} ($MnSO_4$) in a final volume of 0.3 ml in 0.05 M Tris-HCl buffer (pH 7.9). The nuclear sediment preparation was added in amounts of 100–150 μg DNA. Incorporation of labelled precursor was measured as described previously, using a Nuclear Chicago Mark I liquid scintillation counter [5].

2.8. *Determination of ribonuclease activity*

The fractions obtained after sucrose gradient sedimentation were incubated at 37° for 10 min with 5 μg [^{3}H] RNA (rat liver RNA) (specific activity: 234 cpm/ μg). The loss of acid precipitable radioactivity was taken as a measure of RNAase activity.

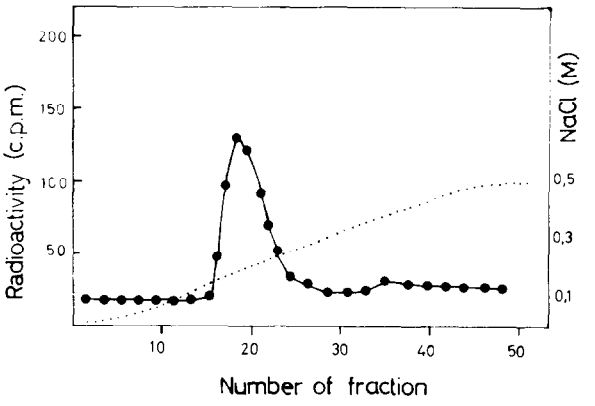


Fig. 1. DEAE-cellulose chromatography of the protein peak obtained by Sephadex (G-25) chromatography of a [^{3}H] cortisol labelled thymocyte nuclear extract. (●—●—●) [^{3}H] radioactivity.

2.9. *Binding of [^{3}H] cortisol to the nuclear sediment in the presence of the nuclear cortisol-binding protein fraction*

Samples of nuclear sediment (150–200 μg DNA) were incubated with cortisol binding protein fractions in the presence of 10^{-9} M [^{3}H] cortisol. The mixtures were adjusted to concentrations of 0.1 M NaCl and the incubation was performed at 3° for 8 hr under continuous shaking. Thereafter the incubation mixture was layered on 5 ml 1.7 M sucrose, containing 0.05 M NaCl and Tris-HCl (pH 7.9) and centrifuged in a SW 65 K Spinco head at 35 000 rpm for 1 hr. The sediments obtained were dried, solubilized with NCS and counted for radioactivity.

Table 1
Binding of [^{3}H] cortisol (10^{-9} M) to thymocyte nuclear proteins obtained by extraction with 0.05 M Tris-HCl (pH 7.9) containing 0.1 M NaCl or 0.3 M NaCl. The binding was determined by equilibrium dialysis for 65 hr at 0°.

	Bound radioactivity ±S.E.	Specific activity (cpm/mg prot.)
0.1 M NaCl extract	408 ± 64	279
0.1 M NaCl sediment extracted with 0.3 M NaCl buffer	262 ± 51	113
0.3 M NaCl extract	813 ± 16	278

3. Results

A cortisol-binding protein can be extracted with 0.3 M NaCl-TEM buffer from isolated thymocyte nuclei and labelled with [^{3}H] cortisol *in vitro*.

As seen in figs. 1 and 2 the cortisol-binding protein is eluted as a single peak after Sephadex G-200 or DEAE-cellulose-chromatography. A molecular weight of approx. 150 000 has been ascribed to this protein on the basis of its elution behaviour on Sephadex G-200 and its sedimentation coefficient on linear 5–20% sucrose gradients. The cortisol-binding protein elutes at 0.2 M NaCl from DEAE-cellulose columns (fig. 1). As shown in fig. 2, DNA-dependent RNA polymerase activity elutes from Sephadex G-200 columns in the excluded fraction together with Dextran blue, well separated from the cortisol recep-

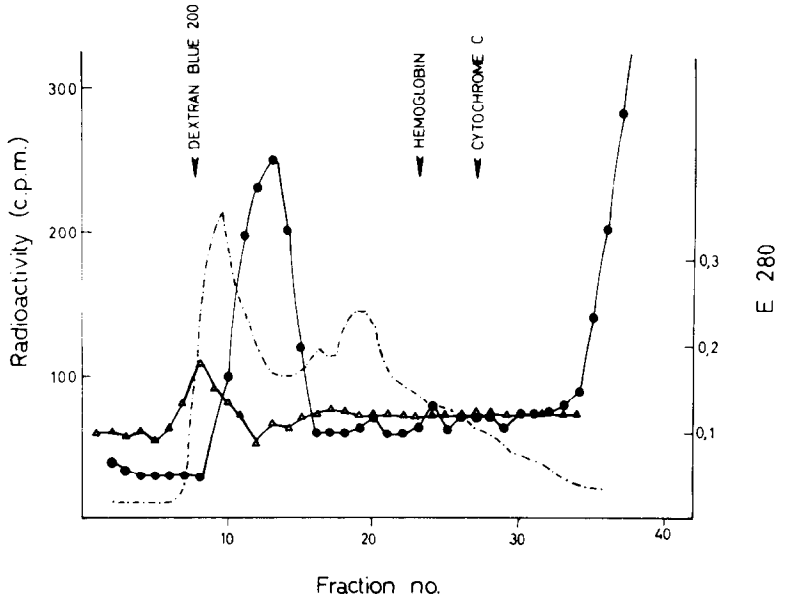
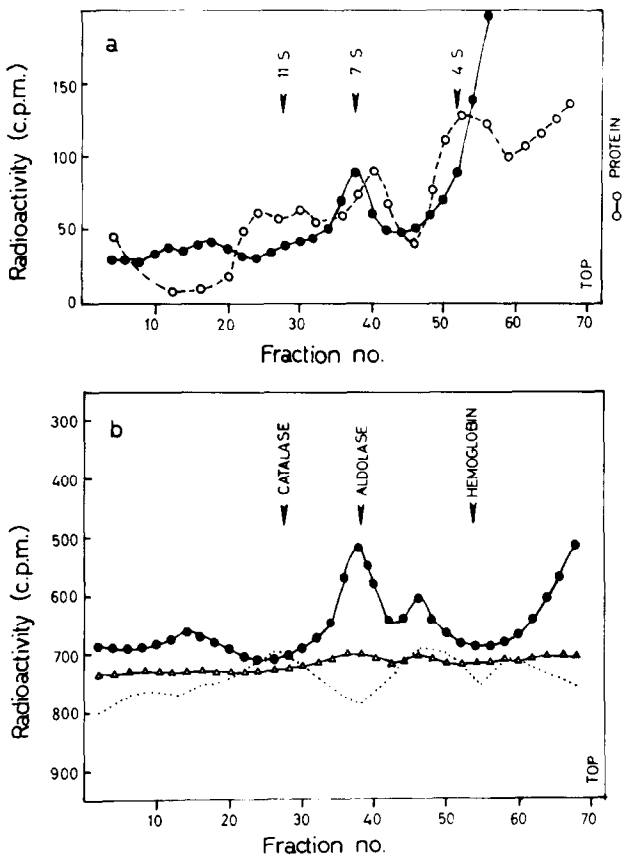


Fig. 2. Chromatography on Sephadex G-200 of *in vitro* [^{3}H] cortisol labelled nuclear extracts derived from isolated thymocytes. The column was equilibrated with 0.3 M NaCl-TEM buffer (pH 8.0). (●—●—●) [^{3}H] radioactivity, (- - - - -) UV absorbancy at 280 nm, (△—△—△) RNA-polymerase activity.

Table 2

In vitro effect of nuclear cortisol binding macromolecular peak in the absence and presence of 10^{-5} M cortisol on the RNA synthesizing capacity of the 0.1 M NaCl nuclear sediment prepared from isolated rat thymocytes.

	Experiments with cortisol + buffer		Experiments with cortisol + macr molecular peak	
	Control	Cortisol	Control	Cortisol
$\bar{X}$ (cpm/mg protein)	516	508	498	408
Per cent modification	–	−1.5	–	−18.1
Number of experiments (n)	8	8	10	10
$\frac{\Sigma(x_i - y_i)}{n}$ ± S.E.	−21.7 ± 17		−97 ± 10	
P	> 0.05		< 0.001	



tor protein. This is important for the transcription experiments described below.

In order to determine whether the cortisol receptor binding protein can be extracted with salt concentrations lower than 0.3 M NaCl we have performed stepwise extraction of purified thymocyte nuclei.

As seen in table 1 a significant part of the recepto is extracted with 0.1 M salt. This is in accord with th observation that cortisol has no significant effect on transcription by the 0.1 M NaCl nuclear sediment (table 2). In order to assess further the functional rol of the cortisol binding nuclear protein we have measured the effect of cortisol on the rate of transcription by nuclei first extracted with 0.1 M NaCl both in the presence and absence of the receptor protein fraction.

Inhibition of RNA synthesis by cortisol is seen only in the presence of the receptor protein fraction

Fig. 3. Effect of fractions from sucrose gradients on RNA synthesis by thymocyte nuclear sediment. a) Linear sucrose gradient centrifugation of the protein peak obtained by Sephadex G-25 chromatography of [^{3}H] cortisol labelled thymocyte nuclear extract. (●—●—●) [^{3}H] radioactivity, (○—○—○) protein pattern (Lowry). b) Fractions from the sucrose gradient were incubated in the absnece (△—△—△) or presence (●—●—●) of 10^{-5} M cortisol with 0.1 M NaCl-nuclear sediment (0.1 ml containing 150–200 μg of DNA) a 0° for 60 min. The preparations were subsequently tested fc RNA synthesizing capacity. Results of the determination of ribonuclease activity are shown by curve (·····).

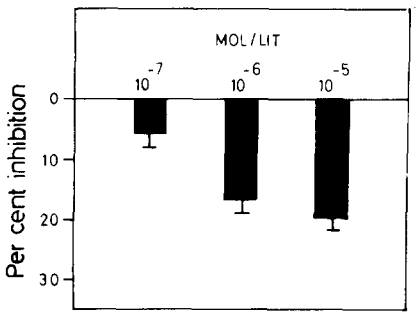


Fig. 4. Influence of cortisol concentrations on the RNA polymerase activity of thymocyte nuclear sediment in the presence of cortisol-binding macromolecular peak obtained by DEAE-cellulose chromatography.

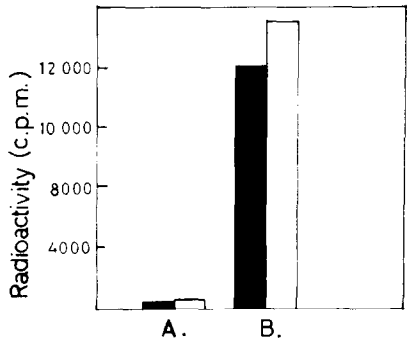


Fig. 5. Association of [^{3}H] cortisol with thymocyte nuclear sediment in the absence or presence of the cortisol-binding macromolecular peak obtained by Sephadex G-200 chromatography. System A consisted of 0.1 M NaCl sediment and 0.3 M NaCl-TEM buffer and [^{3}H] cortisol (10^{-9} M); system B 0.1 M NaCl sediment and 0.3 M NaCl extracted macromolecular peak and [^{3}H] cortisol 10^{-9} M. ■ Counts/min, ▫ counts/min per mg protein.

(table 2). The inhibition amounts to −18% and is highly significant. Similar results have been observed with receptor fractions obtained after separation of cortisol-labelled 0.3 M NaCl nuclear extracts on sucrose gradients (see methods).

The fractions collected after sucrose gradient centrifugation were incubated in the presence or absence of non-labelled cortisol (10^{-5} M) with nuclear sediment and assayed for RNA synthesizing capacity. In the presence of fractions containing the 7 S receptor protein (fig. 3a) an inhibitory effect of cortisol on RNA synthesis can be observed (see fig. 3b). Assay of the various fractions for RNAase activity has demonstrated that the inhibitory effect of the 7 S fraction is not due to unspecific degrading action.

The dependence of the inhibitory effect on the cortisol concentration, under constant receptor-protein concentration, is shown in fig. 4.

In the presence of 10^{-6} M cortisol we could observe a potentiation of the inhibitory effect on transcription (up to 32%) by increasing the receptor-protein concentration.

To gain further information on the mode of action of the receptor protein we have studied its effect on the binding of [^{3}H] cortisol to the 0.1 M NaCl-nuclear sediment. The results are shown in fig. 5. Minimal amounts of free [^{3}H] cortisol bind directly to the 0.1 M NaCl-sediment; however the binding is significantly increased in the presence of receptor protein obtained by Sephadex G-200 chromatography of the 0.3 M NaCl nuclear extract.

4. Discussion

Proteins binding a variety of steroid hormones have been isolated in the last years from the cytosol or the nucleus of target cells [2, 6, 7, 13, 15, 19, 20, 21, 23, 26, 28, 29, 32]. Their possible involvement in hormone action has been indirectly concluded on the basis of several approaches. Thus, receptors are present in hormone-responding tissues and are absent from non-target cells, tumors sensitive to steroid hormones contain hormone-receptors whereas insensitive tumors show a low receptor titer [4, 16]. The dose–response curve of target cells to the hormone shows great similarities to the saturation kinetics of the receptor proteins [27]. Further cells genetically selected to be defective in receptor proteins are also resistant to hormone action [12].

A more direct approach was applied by Beato et al. [6] who showed that in the presence of cytosol binding proteins less cortisol is needed to stimulate RNA synthesis of isolated rat liver nuclei, and by Arnaud et al. [3] who observed stimulation of RNA synthesis of isolated uterus endometrium nucleoli by oestradiol only in the presence of the cytosol receptor for oestradiol.

In this paper we have presented direct evidence for the involvement of a nuclear receptor for cortisol in

hormone action. We could previously show [1, 22] that thymus nuclei respond directly to glucocorticoids with inhibition of RNA synthesis, in the absence of cytoplasmic macromolecules. In a further series of experiments we have demonstrated the presence in the thymocyte nucleus, of a receptor with high affinity for glucocorticoids, having an approximate molecular weight of 150 000 [2]. If this protein is instrumental for the expression of the hormonal effect, then its removal from the nuclear system should make the system insensitive to cortisol. Addition of the receptor to the system should restore its capacity to react to the hormone. This is precisely what we have observed.

As a first step, the nuclear receptor was purified using column chromatography on Sephadex G-200 and DEAE-cellulose as well as sucrose gradient centrifugation. The nuclear receptor was extracted from the nuclei by 0.3 M NaCl which removes almost all of the receptor protein. Nuclei previously treated with 0.1, 0.2 or 0.3 M salt do not react to the presence of cortisol. We have routinely used in our experiments nuclei pretreated with 0.1 M NaCl. As shown above, addition of a receptor fraction obtained either from a DEAE-cellulose column or from a sucrose gradient restores the capacity of the nuclear sediment to respond to cortisol with significant inhibition of RNA synthesis. The possible contamination of the receptor preparations with RNA polymerases, nucleases or proteases has been meticulously controlled and ruled out.

What is the molecular mode of action of the receptor? As shown above the [^{3}H] cortisol-labelled receptor protein binds to the nuclear sediment. This binding is very probably causally connected to the effect of the receptor on transcription. As well documented cortisol preferentially inhibits ribosomal RNA synthesis [1, 10, 22]. The receptor protein by combining with cortisol could be transformed in a factor blocking transcription by RNA polymerase A. In this case binding should be restricted to the ribosomal cistrons. Recent findings [22] have demonstrated that cortisol, parallel to its inhibitory action on nucleolar RNA synthesis, shows a stimulatory action on extranucleolar RNA synthesis which may be causally connected to the inhibition of ribosomal RNA. The role of the receptor in this context should therefore also be considered.

As mentioned above thymus nuclei respond directly to cortisol without the need of cytoplasmic factors. In this respect this system is different from that of oestradiol-uterus one, where the cytosol receptor molecule seems to be absolutely needed for the effect of the hormone on nuclear metabolism.

In the thymus, we visualize the role of cytosol receptors [2] as controlling the rate of entry of the hormone into the nucleus and not taking part in the nuclear events per se. Again this is in contrast to what happens in the uterus where evidence has been presented that the cytosol receptor to oestradiol interacts in the nucleus with nuclear proteins as is the case also for some other systems [6, 19, 20, 26, 32].

It could be that the events in the thymus represent a unique case due to the particular structure of the thymocyte. However, the direct response of isolated nuclei from many tissues to a variety of hormones [9, 13, 18, 19] speaks for the general validity of this finding.

Acknowledgements

We thank Prof. P. Karlson for his encouragement and the Deutsche Forschungsgemeinschaft for financial aid. N.v.d.M. is a scholar of the Deutsche Forschungsgemeinschaft, A.D.A. of the Alexander von Humboldt Stiftung.

References

[1] A.D. Abraham and C.E. Sekeris, Biochim. Biophys. Acta 247 (1971) 562.
[2] A.D. Abraham and C.E. Sekeris, Biochim. Biophys. Acta (1972) in press.
[3] M. Arnaud, Y. Beziat, J.L. Borgna, J.C. Guilleux and M. Mousseron-Canet, Biochim. Biophys. Acta 254 (1971) 241.
[4] J.D. Baxter, A.W. Harris, J.M. Tomkins and M. Cohn, Science 171 (1971) 189.
[5] M. Beato, J. Homoki and C.E. Sekeris, Exp. Cell Res. 55 (1969) 107.
[6] M. Beato, W. Schmid, W. Braendle, D. Biesewig and C.E. Sekeris, Advances in Biosciences, 7. Schering Workshop on Steroid Hormone Receptors, Berlin 7–9, 1970. Pergamon Press. Vieweg. p. 349.
[7] P.A. Bell and A. Munck, Biochem. J. 126 (1972) 118.

[8] K. Burton, Biochem. J. 62 (1956) 315.
[9] C.E. Congote, C.E. Sekeris and P. Karlson, Exp. Cell Res. 56 (1969) 338.
[10] J. Drews and L. Wagner, European J. Biochem. 13 (1970) 231.
[11] T.H. Hamilton, Science 161 (1968) 649.
[12] U. Gehring, G.M. Tomkins and S. Ohno, Nature New Biology 232 (1971) 106.
[13] E.V. Jensen, T. Suzuki, T. Kawashima, W.E. Jungblut, W.E. Stumpf and E.R. de Sombre, Proc. Natl. Acad. Sci. U.S. 59 (1968) 632.
[14] P. Karlson and C.E. Sekeris, Acta Endocr. Copenhagen 53 (1966) 505.
[15] N. Kaiser, A.F. Kirkpatrick, R.J. Milholland and F. Rosen, Federation Proc. 31 (1972) 1191.
[16] A.F. Kirkpatrick, A.F. Milholland and F. Rosen, Nature New Biol. 232 (1971) 216.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[18] J. Lukacs and C.E. Sekeris, Biochim. Biophys. Acta 134 (1967) 85.
[19] W.I.P. Mainwairing and B.M. Peterken, Biochem. J. 125 (1971) 285.
[20] B.W. O'Malley, T.C. Spelsberg, W.T. Schrader, F.C. Chitil and A.W. Steggles, Nature 235 (1972) 141.
[21] J. Mester and E.E. Baulieu, Biochim. Biophys. Acta 261 (1972) 236.
[22] N. van der Meulen, R. Marx, C.E. Sekeris and A.D. Abraham, Exp. Cell Res. (1972) in press.
[23] A. Munck, Persp. Biol. Med. 14 (1971) 265.
[24] T.A. Musliner and G.J. Chader, Biochim. Biophys. Acta 262 (1972) 256.
[25] S. Nakagawa and A. White, Federation Proc. 30 (1971) 944.
[26] G. Raspé (ed.), Advances in Biosciences, 7. Schering Workshop on Steroid Hormone Receptors, Berlin 7–9, 1970. Pergamon Press, Vieweg.
[27] H.H. Samuels and G.M. Tomkins, Federation Proc. 30 (1971) 1048.
[28] B.P. Schaumburg, Biochim. Biophys. Acta 214 (1970) 520.
[29] B.P. Schaumburg, Biochim. Biophys. Acta 263 (1972) 414.
[30] C.E. Sekeris, Biochem. J. 124 (1971) 43 p.
[31] J.R. Tata, Progr. Nucleic Acid Res. Mol. Biol., Vol. 5 (Acad. Press, New York–London, 1966) p. 191.
[32] C.R. Wira, H. Rochefort and E.E. Baulieu, Acta Endocr. Copenhagen 67, Suppl. 153 (1971) 233.